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High Anti-*Cryptosporidium parvum* IgG Seroprevalence in HIV-Infected Adults in Limpopo, South Africa

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Abstract. A seroepidemiological study was performed to determine the seroprevalence of *Cryptosporidium* in human immunodeficiency virus (HIV)-infected adults and local university students in the Limpopo Province, South Africa. Using a custom anti-*C. parvum* immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA), the seroprevalence of *Cryptosporidium* was found to be significantly higher (75.3%; 146 of 193) in HIV-infected individuals compared with student volunteers (32.8%; 19 of 58) ($P < 0.001$). A more recent diagnosis of HIV was associated with anti-*C. parvum* IgG seropositivity, as was lower weight among HIV-infected women. This is the first seroepidemiologic study of *Cryptosporidium* in rural South Africa, and it shows high endemicity among the HIV-infected population. In addition to raising the possibility of significant *Cryptosporidium*-related morbidities, this finding reveals that in Limpopo and perhaps in other low-income, rural populations, interrupting waterborne pathogen transmission will require strategies effective against environmentally hardy parasites such as *Cryptosporidium*.

INTRODUCTION

Cryptosporidium is a well-known cause of waterborne diarrhea in low-income countries. Infection is particularly severe in immunocompromised hosts, namely malnourished children who suffer from repeat infections,[1] persistent diarrhea,[2] childhood stunting,[3–5] and individuals living with advanced human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) who manifest chronic and even severe cholera-like diarrhea.[6,7] In resource-limited settings, co-infection with *Cryptosporidium* and other diarrheal pathogens in patients with HIV/AIDS increases morbidity and mortality, despite the initiation of anti-retroviral therapy, adequate serum anti-retroviral levels, and an appropriate immunological response to therapy.[8–10] The current therapeutic options for *Cryptosporidium* infection are only marginally effective, and for highly endemic regions, innovative technologies are needed. In a recent pilot study that introduced ceramic water filters into the homes of people living with HIV in the Limpopo province, we found reduced diarrheal events and decreased stool prevalence of *Cryptosporidium* in the intervention households[11]; we thus sought to better describe *Cryptosporidium* exposure among this population.

The Limpopo province of northern South Africa (population 5.4 million gross domestic product US$11,000) has a high HIV endemicity and presumptively high *Cryptosporidium* exposure. The majority of the region is rural, and water quality shows that although access to an upgraded basic water supply in the region has improved, fecal coliform counts of groundwater boreholes, particularly community boreholes during the rainy season, exceed both the South Africa and World Health Organization's (WHO) benchmark limits.[12,13]

We have previously reported that the stool-shedding prevalence of *Cryptosporidium* shedding by polymerase chain reaction (PCR) in Limpopo is 13% among HIV-infected adults hospitalized with chronic diarrhea (25.5% of among all hospitalized patients). Whereas others had reported *Cryptosporidium*-positive stools were present in 73.6% of children in Uganda, only 17.9% of school children in the Vhembe District were PCR-positive for *Cryptosporidium*.[14–16] We also found that 50% of those hospitalized, 50–59 years of age in Limpopo, had stool PCR positive for *Cryptosporidium*. Thus, it appeared that *Cryptosporidium* exposure was present in all age groups, and although highly specific for incident infection, the cross-sectional stool analysis may have underestimated exposure possibly caused by temporal and seasonal variations in *Cryptosporidium* transmission.[17] To explore this possibility, we performed a serological survey using an easy-to-perform custom anti-*Cryptosporidium* immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) to enhance knowledge of *Cryptosporidium* exposure in Limpopo.[18,19]

We assayed banked plasma samples (stored at −70°) from 194 serologically HIV-seropositive adults who presented at seven provincial health care facilities (including both high population density semi-urban communities: Bela Bela, Polokwane, and Thohoyandou, and low density population rural communities: Madimbo, Makulleke, Nithaveni, and Mititti) in Limpopo Province, South Africa in 2007, and 58 fresh plasma samples (recovered from whole blood centrifuged at 2,000 rpm for 10 minutes within 4 hours of collection) from student volunteers collected in August 2008.

To detect anti-*Cryptosporidium* IgG in serum, we used our previously published custom ELISA that had a reported sensitivity of 94% compared with stool microscopy using the previously validated cutoff of $(OD_{sample}/OD_{negative\ control}) \geq 1.8$[20] (expressed as "ELISA units" [EU]). *Cryptosporidium* parasite extract (PE) was prepared from a stock of 1×10^9 purified *C. parvum* oocysts (Iowa isolate; Waterborne, Inc., New Orleans, LA). Washed oocysts were resuspended in carbonate buffer (pH 9.6) and disrupted using a Branson sonifier cell disrupter (model W140D; Heat System-Ultrasonics, Inc., Plainview, NY) until > 90% oocyst disruption was confirmed by examination with a hemocytometer. The resulting PE was coated onto 96-well plates at a final concentration of 0.2 μg/100 μL/well and incubated in carbonate-bicarbonate coating

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buffer overnight at 4°C. Plates were washed three times with wash solution (Kirkegaard & Perry Laboratories, Inc. [KPL, Inc., Gaithersburg, MD]) to remove any uncoated proteins, and wells were then blocked overnight with 1% phosphate buffered saline-bovine serum albumin at 4°C(KPL, Inc.) and washed before addition of 50 μL of plasma (1:32 dilution). Following a 1-hour incubation at 37°C, the patient plasma sample was washed, and 50 μL of alkaline phosphatase-conjugated goat anti-human IgG antibody (1:1,000) (KPL, Inc.), was added and incubated at 37°C for another 1 hour. Following repeat washing, *p*-nitrophenylphosphate substrate (Sigma -Aldrich Diagnostics, St. Louis, MO) was added for the final reaction step.[20] Absorbance was read at 405 nm on a spectrophotometer beginning 5 minutes after addition of the substrate and at 3–5-minute intervals thereafter for up to 60 minutes. Statistical analyses were performed using χ^2 and Mann-Whitney *t*-test when applicable on IBM SPSS Statistics v. 20 (IBM Corp., Armonk, NY) or GraphPad Prism 5.0 d (GraphPad Software, Inc., La Jolla, CA) for Mac OSX. Missing data were categorized as "unknown" for each respective variable. A $P < 0.05$ was considered statistically significant. The optical density of internal negative and positive controls ranged from 0.135–0.256 (mean ± SD; 0.1939 ± 0.039 for all plates) and 0.5375–0.9061 (mean ± SD = 0.7826 ± 0.2050 for all plates), respectively. Under varying laboratory conditions (including humidity and temperature) there was 4–50% variability among 18 samples repeated on separate days (Student's paired *t* test, $P = 0.0683$).

Among patients in the HIV-clinic cohort, the median normalized Elisa Units (EU) values (EU-1.8) (range = –1.006 to 4.566; median ± interquartile range (IQR) = 0.4591 ± 1.159; $N = 194$; 75.3% positive) were greater than EU values among the student cohort (range = –1.190 to 1.228; median ± IQR = –0.1571 ± 0.7517; $N = 58$; 32.8% positive) ($P < 0.0001$) (Figure 1A). The student cohort was significantly younger than the HIV-clinic cohort (median ± IQR = 22.0 ± 3 versus 34.50 ± 14, respectively, $P < 0.05$ Mann-Whitney). Within an age-matched subgroup 35.7% ($N = 42$) of the student cohort and 94.4% of HIV-clinic cohort ($N = 18$) were positive ($P < 0.0001$) (Figure 1B). Anti-*C. parvum* IgG seropositivity in the Vhembe district ranged from 58% in communities in the immediate vicinity of Thohoyandou to 80–100% in small rural clinics in northern Vhembe (ns). The Polokwane and Bela-Bela vicinity showed similar seropositivity, 70.0% and 64.4%, respectively (ns) (Supplemental Figure 1). The widespread and nearly universal exposure to *Cryptosporidium* among those living with HIV in Limpopo is strikingly greater than we had previously reported using stool diagnostics.[14,15] The findings raise important concerns regarding the high exposure to waterborne pathogen exposure in this HIV-positive population. Although this report does not enable us to infer demographic differences that account for the higher exposure in the HIV-positive cohort compared with the student cohort, our observation underlines the need for carefully designed future case-control analyses that may identify particular associations or behaviors that influence exposure to *Cryptosporidium* or other waterborne pathogens in the region.

We performed a hypothesis-generating preliminary univariate analysis on the HIV-clinic cohort subjects to evaluate potential exposure risks (Table 1). Anti-*C. parvum* IgG seropositive subjects were more likely to have received a diagnosis of HIV within 6 years of sample collection (78.1%,),

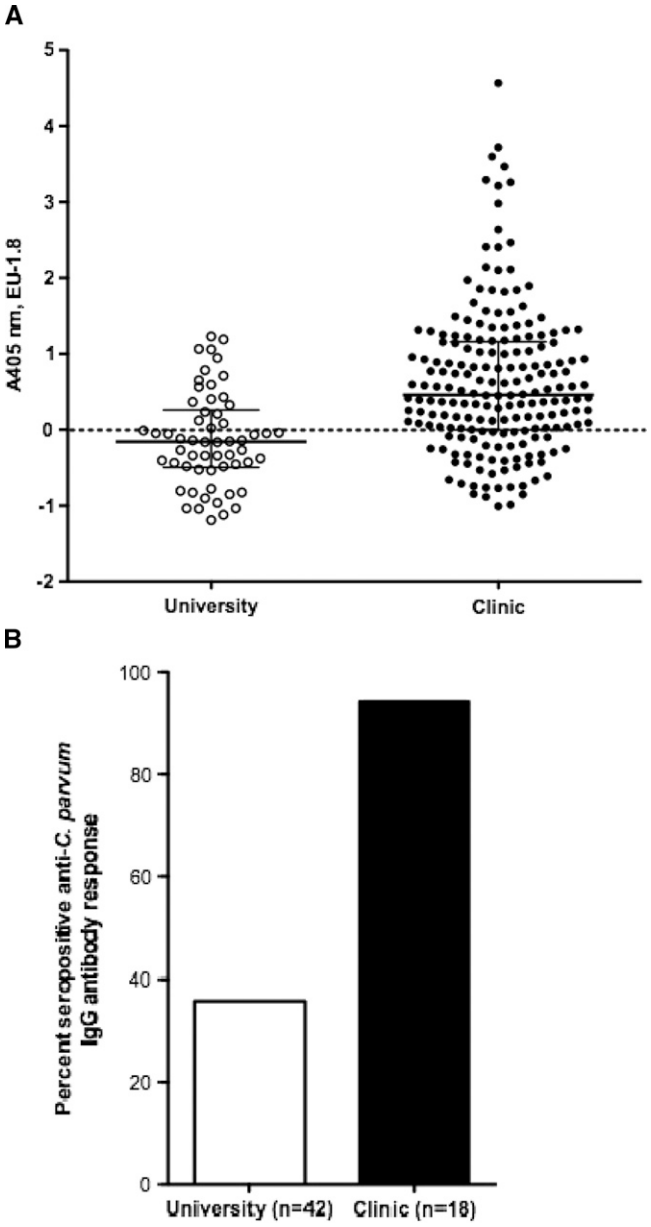

FIGURE 1. Anti-*Cryptosporidium parvum* immunoglobulin G (IgG) antibody responses to *C. parvum* parasite extract among university student and human immunodeficiency virus (HIV)-clinic cohorts. (**A**) Normalized anti-*C. parvum* IgG enzyme-linked immunosorbent assay (ELISA) Units (EU-1.8) such that values > zero are seropositive. Among university students, 32.8% ($N = 58$) were positive (median ± interquartile range [IQR] = –0.1571 ± 0.7517) and 75.3% ($N = 194$) patients from HIV clinics were positive (median ± IQR = 0.4591 ± 1.159) ($P < 0.0001$, Mann-Whitney). (**B**) Within an age-matched sub-cohort of the two groups, 35.7% ($N = 42$) of university students and 94.4% ($N = 18$) of HIV-clinic patients were positive ($P < 0.0001$, χ^2).

whereas 58.4% of anti-*C. parvum* IgG seronegative subjects were diagnosed with HIV 7 or more years prior ($P = 0.040$). There was no significant difference in anti-*C. parvum* IgG serostatus among patients with more advanced WHO Stage disease ($P = 0.204$). Of those reporting a monthly income, the highest proportion of anti-*C. parvum* IgG seropositive results were in those receiving < 10,000 R/month or on subsidized income, 76% (118 of 154); however, the prevalence of anti-*C.*

TABLE 1

Univariate analysis of the human immunodeficiency virus (HIV)-clinic cohort by anti-*C. parvum* serostatus*

Characteristic	Seronegative ($N = 48$), N (%)		Seropositive ($N = 146$), N (%)		Total ($N = 194$), N (%)		P value (χ^2)
Age							0.074
< 25 years	6 (12.5)		30 (20.5)		36 (18.6)		
26–35 years	15 (31.3)		52 (35.6)		67 (34.5)		
36–45 years	18 (37.5)		34 (23.3)		52 (26.8)		
> 45 years	3 (6.25)		24 (16.4)		27 (13.9)		
NR†	6 (12.5)		6 (4.1)		12 (6.2)		
Sex							0.683
Male	10 (20.8)		28 (19.2)		38 (19.6)		
Female	34 (70.8)		112 (76.7)		146 (75.2)		
NR†	4 (8.3)		6 (4.1)		10 (5.2)		
Duration of HIV							0.040
1–3 years	13 (27.1)		58 (39.7)		71 (36.6)		
4–6 years	15 (31.3)		56 (38.4)		71 (36.6)		
7–10 years	13 (27.1)		17 (11.6)		30 (15.4)		
> 10 years	3 (6.3)		2 (1.4)		5 (2.6)		
NR†	4 (8.3)		13 (8.9)		17 (8.8)		
WHO HIV/AIDS stage							0.204
Asymptomatic/primary HIV	0 (0.0)		5 (3.4)		5 (2.6)		
I	8 (16.7)		36 (24.7)		44 (22.7)		
II	10 (20.8)		25 (17.1)		35 (18.0)		
III	13 (27.1)		32 (21.9)		45 (23.2)		
IV	5 (10.4)		6 (4.1)		11 (5.7)		
NR†	12 (25)		42 (28.8)		54 (27.8)		
Monthly income							0.803
None	10 (20.8)		28 (19.2)		38 (19.6)		
Agricultural	2 (4.2)		7 (4.8)		9 (4.6)		
Subsidized grant	9 (18.8)		21 (43.8)		30 (15.4)		
< 1,000 Rand (R)‡	12 (41.7)		48 (32.9)		60 (30.9)		
1,000–10,000 R	3 (6.3)		14 (9.6)		17 (8.8)		
> 10,000 R	3 (6.3)		3 (2.1)		6 (3.1)		
Unknown, used	1 (2.1)		3 (2.1)		4 (2.1)		
Unknown, unemployed	1 (2.1)		3 (2.1)		4 (2.1)		
NR†	7 (14.6)		21 (43.8)		26 (13.4)		
Weight quartile	M (10)	F (31)	M (27)	F (97)	M (37)	F (128)	< 0.001 (F)
< 53 kg	1 (10.0)	5 (16.1)	5 (18.5)	28 (28.9)	6 (16.2)	33 (25.8)	
53–59 kg	3 (30.0)	5 (16.1)	7 (25.9)	24 (24.7)	10 (27.0)	29 (22.7)	
60–66 kg	4 (40.0)	10 (32.3)	7 (25.9)	25 (25.8)	11 (29.7)	35 (27.3)	
> 67 kg	2 (20.0)	11(35.5)	8 (29.6)	20 (20.1)	10 (27.0)	31 (24.2)	

*"Seropositive" and "Seronegative" refer to anti-*Cryptosporidium parvum* immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) result. Data are stratified into quartiles where appropriate and P values determined by χ^2. M = male, F = female.

†NR = no response.

‡1,000 Rand ≅120 US$.

parvum IgG seropositivity was not statistically significant across income brackets ($P = 0.803$). Although only preliminary, this analysis conveys important information for local providers. *Cryptosporidium* exposure is common across the spectrum of HIV stages of disease (i.e., not just those with advanced disease). Recent diagnosis of HIV can be interpreted as a surrogate for access to health care and may represent a critical opportunity to evaluate and educate regarding safe water-sanitation practices among this population. Finally, patients in all income brackets in this population have evidence of exposure to *Cryptosporidium*. Given the known high coliform counts in the community ground water in Limpopo[21] this additional information that highlights the high prevalence of protozoal pathogens is critical to the design of local public health interventions that emphasize point-of-use water purification strategies and sanitation improvements that strive to limit acquisition of waterborne pathogens both in Limpopo and elsewhere.

In conclusion, we have used a simple and low-cost custom ELISA to better define *Cryptosporidium* exposure in the Limpopo Province of South Africa. We report that exposure is highly endemic among HIV-co-infected individuals in the region. Although more comprehensive studies are needed to clarify specific risk factors and disease-associated morbidities in this population, our observations emphasize an example of the importance of establishing baseline knowledge of the exposure to pathogens such as *Cryptosporidium* in populations who need improved water–sanitation technologies. Knowledge of the high exposure to *Cryptosporidium* infection in this population informs clinical decision making, the development of public health strategies that are inclusive of technology that interrupts *Cryptosporidium* transmission, and future investigations to gauge the effectiveness and sustainability of water–sanitation interventions.

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